BLOOD COAGULATION IN ARTHROPODS. III. REACTIONS OF INSECT HEMOLYMPH TO COAGULATION INHIBITORS OF VERTEBRATE BLOOD [1]

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As shown in previous studies by means of the phase contrast microscope (Grégoire and Florkin, 1950; Grégoire, 1951a), hemolymph coagulation in a number of insects appears to be a continuous process, initiated by alterations taking place rapidly in a category of highly labile hemocytes (coagulocytes). These alterations are followed by the development of islands of coagulation in the surrounding plasma. Various degrees of extension of the process occur in different species of insects, from a general clotting, macroscopically visible, to a limited reaction, consisting of a few microscopic islands of coagulation, centered by these hemocytes and scattered in an apparently fluid hemolymph.

The aim of the present study was to investigate by means of the phase contrast microscope the reactions of the insect hemolymph to substances or physical conditions inhibiting the coagulation process in vertebrates and other invertebrates. Insects, chiefly Orthoptera, characterized by a conspicuous clotting process, were selected as experimental material.

The appearance or the absence of the early alterations taking place in the category of highly labile hemocytes and in the neighboring plasma was found to be an expedient test for the evaluation of the degree of interference with the coagulation process brought about by various compounds and experimental conditions.

Material and methods

The species of insects used for the present investigations were *Locusta viridissima* (great green grasshopper), *Chorthippus* sp. (short-horned grasshopper), *Gryllulus domesticus* (house cricket), *Periplaneta americana* L. (cockroach), *Blabera gigantea* (South American cockroach), *Mantis religiosa* (praying mantis), *Gryllotalpa gryllotalpa* (mole-cricket), *Carausius morosus* (stick insect) (Orthop-

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tera); *Forficula auricularia* (earwig) (Dermaptera); *Nepa cinerea* (water-scorpion), *Lethocerus cordofanus* Mayr (Belostomatidae, Belgian Congo) (Hemiptera); *Meloe proscarabaeus* (oil beetle), *Tithoes frontalis* (Longicorn from Belgian Congo) (Coleoptera); and *Diprion pini* (Hymenoptera).

Thirty-three substances were tested. Most of them are anticoagulants on vertebrate blood *in vivo* or *in vitro*. These substances were arranged in the summarized report of the data below according to MacIntosh's (1949) classification of their mode of action on vertebrate blood. In addition, the disodium salt of ethylene diamine tetraacetic acid (Sequestrene NA 2: Alrose Chem. Co.: Dyckerhoff, Marx and Ludwig, 1942; Proescher, 1951), Apikur (Lenggenhager, 1949), hexamethylene-glycol (Foster, Samsa, Shulman and Ferry, 1951; Shulman and Ferry, 1951), the sodium salt of sulfated polygalacturonic acid methyl ester methyl glycoside or Treburon, Ro2–3053 (Mangieri, Engelberg and Randall, 1951), recently reported as anticoagulants of vertebrate blood, were also studied. We were unable to obtain hirudin and novirudin.

For study the reagents were dissolved or diluted at various concentrations with Meisenheimer's fluid, a special Ringer solution for insects (Bi-distilled water: 1000 gm.; NaCl: 7.5 gm.; $NaHCO_3$: 0.1 gm.; KCl: 0.2 gm.; $CaCl_2$: 0.2 gm.) This fluid was found to be a relatively good preservative of the cellular structures and did not interfere with the reactions. Distilled water was also used. The pH of the solutions was controlled before use.

Droplets of hemolymph issuing from the severed end of an antenna or a leg were allowed to fall in approximately the same volume of solution placed in the middle of a standard microscopical slide (Gold Seal glass). Preparations were also made by dipping the severed antenna directly into the solution. The mixture of hemolymph and solution, approximately 0.005 to 0.01 cc. in total volume, was homogenized by gentle lateral shakings of the slide. A thin Gold Seal coverglass, wet in its middle part with a droplet of the solution, was thus gently placed down onto the slide, droplet against drop of mixture. Spontaneous spreading gave a film of suitable thinness for observation. With this procedure, the labile hemocytes were generally protected from contact with glass surfaces before being thoroughly mixed with the solution of the inhibitor.

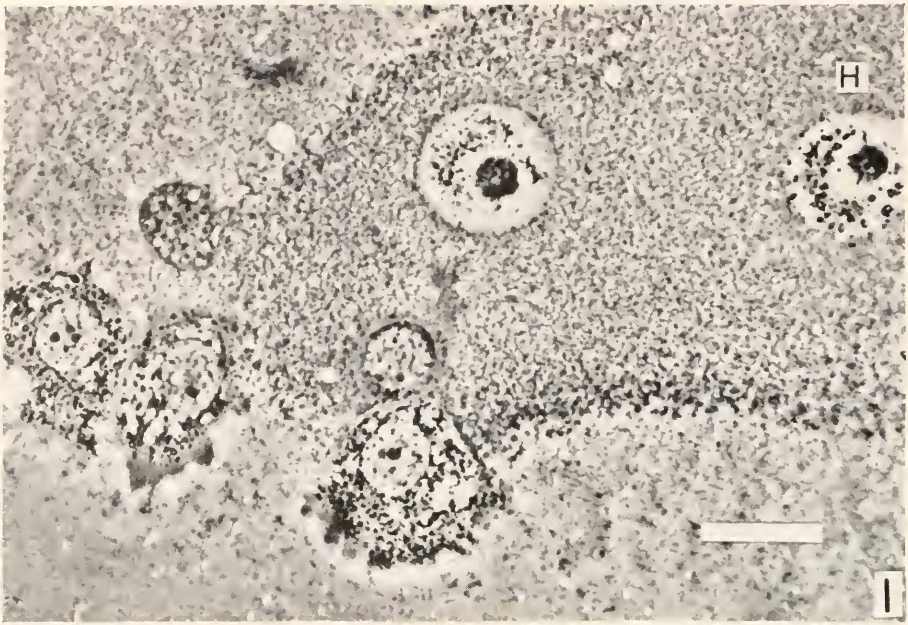
Control preparations were made with Meisenheimer's fluid alone at various pH values in order to appreciate the part played by the dilution of the hemolymph and by the pH in the modifications of the coagulation pictures.

Reactions of the hemolymph to contact with surfaces of various plastics (cellophane, acetophane, plexiglass, Lucite) or with glass made hydrophobic by coating with paraffin, formvar, parlodion and silicone G. E. Drifilm No. 9987, were also studied.

Silicone coatings of glass were prepared according to the standard technique used with vertebrate blood, Jaques *et al.* (1946). The preparations, stored in moist chambers, were observed during several hours.

Results

Nine hundred tests approximately were performed with the substances listed in the summarized report of the data (see below).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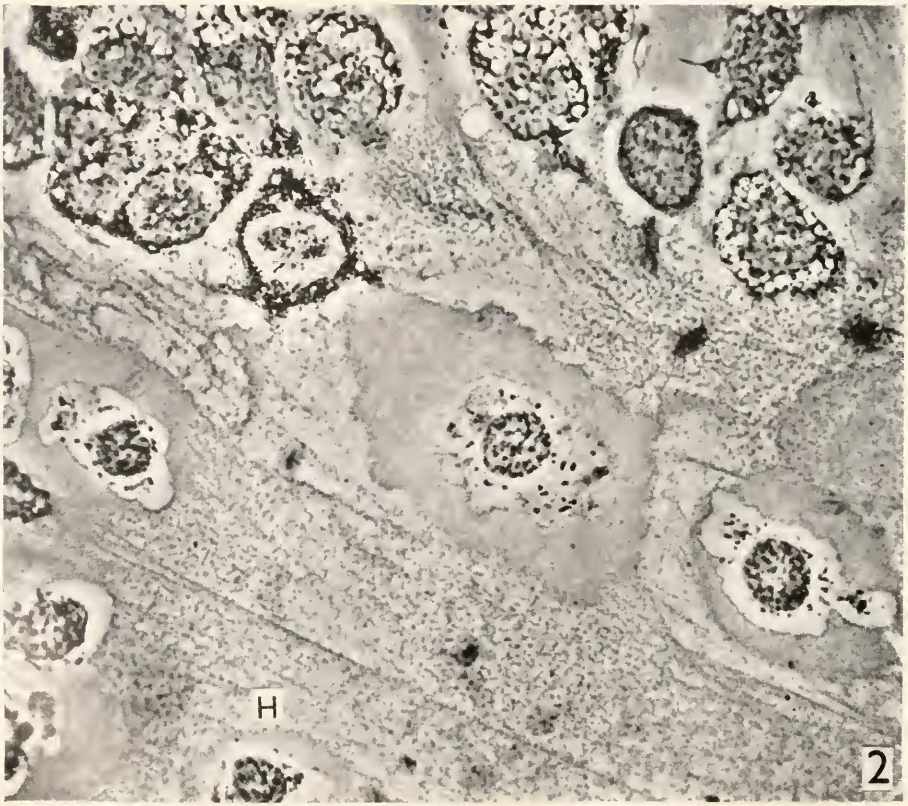
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Control of the effects of dilution

The degree of dilution of the hemolymph with the Meisenheimer's Ringer used in the present experiments (50%) did not interfere with the process of coagulation. The successive stages previously described in the normal hemolymph were also observed to take place after dilution. The highly labile hemocytes were characterized by a relatively small, sharply outlined nucleus, a pale hyaline cytoplasm, in which a few black granular particles were scattered. Within a few seconds, these hyaline hemocytes underwent a succession of intense modifications in their cytological structure: in the mole-cricket, for instance, the changes consisted of a rapid expansion of the cytoplasmic substance, with occasional beam-like bulging of cytoplasmic blisters and blebs (see Grégoire, 1951a; Figs. 2, 3, 6, 7, 8, 12). These alterations were accompanied or followed by the appearance in the surrounding plasma of a thin fog, developing into a circular cloud made of granular particles and progressively increasing in amount and in density. Variable extension of the process and organization of the coagulum into a delicate meshwork of fibrils took place subsequently (Figs. 1 and 2).

The difference between undiluted and diluted materials consisted chiefly of a decrease, proportional to the degree of the dilution, in the size of the islands and in the density of the coagulating materials (see Grégoire, 1951a; Figs. 1 and 2).

By spreading out between slide and coverslip, the diluted coagulum often assumed the shape of thin granular and elastic veil-like structures, a type of picture described elsewhere (Grégoire, 1951a; Figs. 24 and 26) in insect species belonging to various orders of insects (Coleoptera, Lepidoptera). The laying of the coverglass onto the slide often brought about a drawing of the coagulum into strings or threadlike fibrils. In the species used in the present studies, veils and strings were subsidiary morphological aspects induced by mechanical agencies.

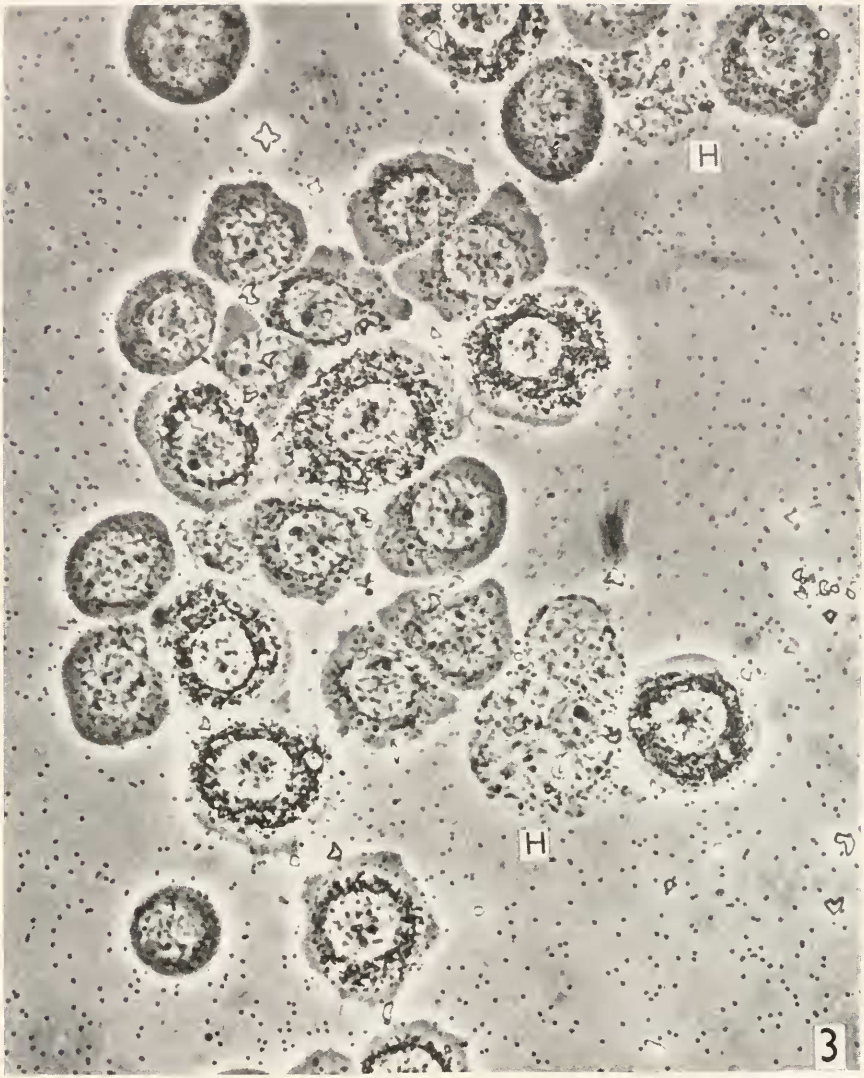
In most preparations, unless otherwise specified, one drop of hemolymph is added to one drop of a solution in Meisenheimer's Ringer of the substance to be tested, lying on a microscopical slide. The mixture, homogenized by lateral shaking of the slide, is allowed to spread out as a thin film between slide and coverglass.

All the microphotographs were taken by phase contrast microscopy (P. C. M. *Wild* M/10, Heerbrugg, Switzerland, with the objectives Ph 7/0.20 and Ph 40/0.66; Microcamera Leitz/Makam) and subsequently enlarged. The scale on the pictures represents 20 microns.

Variations in size of the hemocytes, as observed in the present pictures, are unreliable: they depend chiefly on adhesiveness factors varying with the amount of hemolymph, the area of spreading out, the thickness of the film and the local pressure of the coverglass onto the slide.

FIGURE 1. Mole-cricket (*Gryllotalpa gryllotalpa*, Orthopt.) Undiluted hemolymph, 20 minutes after withdrawal. Two hyaline hemocytes (H) are seen surrounded by a coagulation island. Extension of the process of coagulation at the upper left and lower right of the picture. Three granular hemocytes and two small round cells are being passively embedded in the extending granular coagulum. In the control preparations of the dilution factor, identical reactions can be observed, but some of the coagulation islands are of smaller size than in undiluted preparations (compare with Grégoire, 1951a, Figs. 1 and 2). × 800.

FIGURE 2. American cockroach (*Periplaneta americana* L., Orthopt.). Undiluted hemolymph, 12 minutes after withdrawal. Dense coagulation islands around several hyaline hemocytes. In the lower half of the picture, area of extension and of organization of the coagulum into a granular meshwork. About 13 elements, belonging to the other categories of hemocytes (granular, small round cells and transition forms) are passively embedded in the coagulum. × 800.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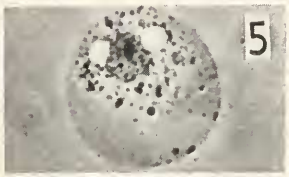
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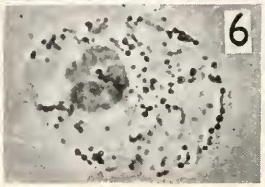
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Control of the factor pH of the solution investigated

Dilution of the hemolymph with Meisenheimer's fluid, brought to different pH covering the ranges of pH recorded in most solutions of the substances investigated, did not interfere with the process of coagulation. However, when hemolymph was diluted with Meisenheimer's fluid at pH 0.6 and 1.20, precipitates and a decrease in the amount of the coagulating materials were occasionally recorded. On the other hand, dilution with Meisenheimer's fluid at pH 9.45 appeared to induce an increase in the amount of the coagulum. This effect of alkalinity has already been pointed out (Muttkowski, 1924; Beard, 1950).

Microscopic aspects of the coagulation inhibition

The alterations taking place in the presence of potassium oxalate (at the concentration of 0.2% up) may be selected for the description of the total inhibition of the process of coagulation brought about by various compounds.

When the hemolymph was mixed with a solution of this substance, the hyaline hemocytes failed to undergo the alterations observed with the normal or the diluted blood. They appeared as pale spherical or oval cucumber-like bodies, loosely fixed to the glass or floating freely (Figs. 3 to 6, 15, 20 and 21).

Occasional rupture of these hyaline hemocytes, discharge of their cytoplasmic substance or granules did not bring about any change in their surrounding plasma; neither did contact of these elements (Figs. 18, 19) with foreign bodies, which in the normal blood rapidly induced the local formation of a coagulation island.

The other categories of hemocytes (small round or so-called stem cells, various kinds of granular hemocytes and transition forms), scattered or agglutinated in clusters at random, appeared also as spherical, oval or polygonal elements (Fig. 3). They occasionally spread out moderately and sent short spiky pseudopodia or bulging blebs (Fig. 4).

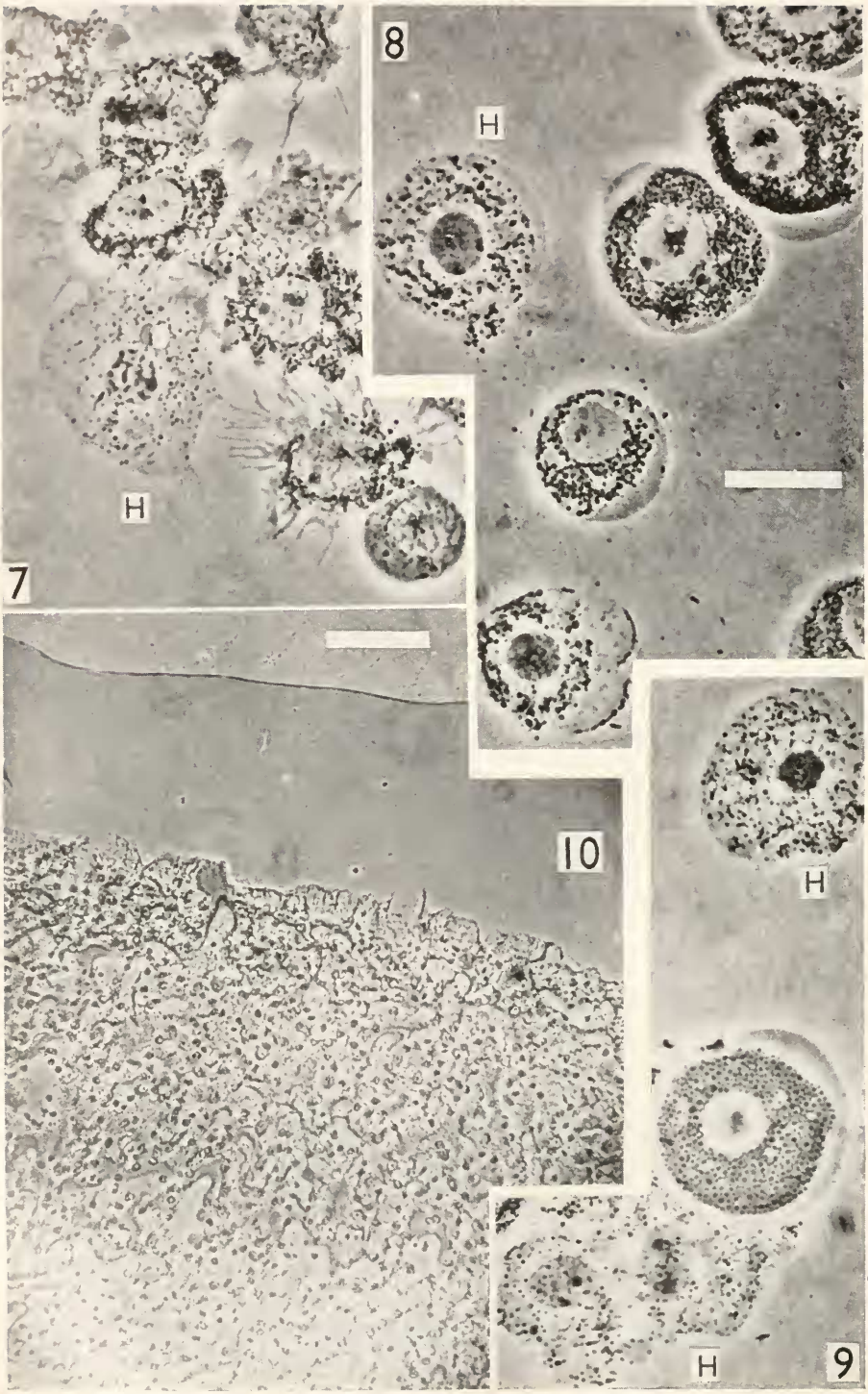
The oxalate type of reaction was observed in mixtures of equal amounts of hemolymph and solutions of potassium oxalate (0.2% up; Figs. 3 to 6, 11, 15, 20 and 21), sodium citrate (1%; Fig. 7), saturated magnesium sulfate, Sequestrene NA 2 (0.1% up; Figs. 12, 22, 23); of organic esters of sulfuric acid: Treburon [2] (1% up), Suramin (Belganyl: 0.1% up; Fig. 13); Bayer 205 (Germanine:

[2] A 0.1% solution of benzophenol, corresponding to the amount of antiseptic contained in the Treburon and heparin vials used, did not interfere with the coagulation process, in control preparations.

FIGURE 3. *Gryllotalpa*. Twenty-nine minutes after addition of three drops of hemolymph to one drop of a 1% solution of potassium oxalate (pH 7.60). No plasma reaction around two hyaline hemocytes. The other categories of cells are loosely agglutinated. Some send out thin spiky pseudopodia. The black dots in the plasma are precipitates of calcium oxalate. × 800.

FIGURE 4. *Gryllotalpa*. Hemolymph added to a 1% solution of potassium oxalate (pH 7.60) 80 minutes after mixture. No plasma reaction around a spread hyaline hemocyte. Two granular hemocytes with bulging blisters. × 800.

FIGURES 5 AND 6. *Gryllotalpa*. Hemolymph added to a 1% solution of potassium oxalate (pH 7.60) 28 and 60 minutes after mixture. No plasma reaction around two swollen hyaline hemocytes. Owing to the active motion of the intra-cytoplasmic granules, these structures lack sharpness in the picture. × 800.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0.2% up), Chicago Blue 6 B (0.1% up, with *Periplaneta* hemolymph; 1% up, with *Gryllotalpa* hemolymph; Fig. 14), Liquoid (1% up; Fig. 19), Chlorazol fast pink BKS (0.02% up); of organic bases and basic dyes: Methylene blue (2%), Janus green B (0.02% with *Periplaneta* hemolymph, 0.2% with *Gryllotalpa* hemolymph); of reducing substances: sodium bisulfite (2% up), sodium thiosulfate (2% up; Figs. 17 and 18), sodium hydrosulfite (3%). With 0.1% solutions of potassium oxalate and concentrations lower than 1% of sodium citrate (0.3%, 0.4%), definite inhibitory effects were recorded, but they were not constant.

Absence of coagulation was recorded with a 10% solution of Lanthanum chloride, with solutions of cocaine hydrochloride (1% up; Figs. 8 and 9) and with a 10% solution of DDT.

In mixtures of hemolymph with solutions of Heparin,[2] diethylamine, methyl violet, cysteine hydrochloride, glutathione, l-ascorbic acid, peptone, sodium taurocholate, Apikur, hexamethylene-glycol, a great deal of variation was recorded in the reactions, and anticoagulant effects of the oxalate type could not be consistently observed.

In several tests, there was a definite lack of coagulation, especially when using heparin or solutions at high concentration of acidified diethylamine (10%) alkalinized glutathione (10%), alkalinized cysteine hydrochloride (10%), peptone (10% up) and sodium taurocholate (16% up).

On the other hand, a 10% solution of cysteine hydrochloride (pH 0.60) seemed to increase the rapidity of initiation of the process of coagulation and the amount of coagulum.

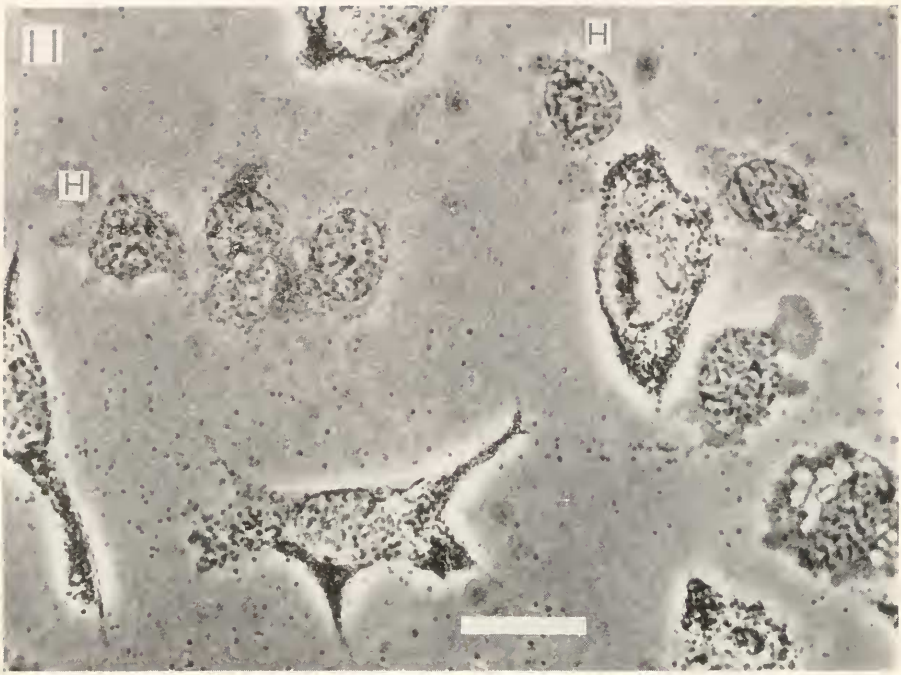
In other tests performed with the same substances, the process was not totally prevented, as in the oxalate type, but was definitely reduced; thin coatings of coagulating substance developed around a limited number of hyaline hemocytes; the clotted materials were decreased in amounts when compared with the corresponding formations in the dilution controls. Extension of the coagulation process from around the coagulation islands did not occur or was limited.

At the concentrations used in the present work, various solutions of saccharose, tricalcium phosphate, protamine, nucleic acid, sodium nucleinate did not interfere with the process of coagulation. However, pictures of inhibition were occasionally observed with solutions of sodium nucleinate (Fig. 16).

FIGURE 7. *Gryllotalpa.* Hemolymph added to a 1% solution of sodium citrate (pH 7.58) two hours after mixture. No plasma reaction around the hyaline hemocyte, containing intracytoplasmic granules in intense motion. Spreading out of the hyaloplasm of the granular hemocytes. × 800.

FIGURES 8 AND 9. *Gryllotalpa.* Ten and five minutes after addition of hemolymph to a 1% solution of cocaine hydrochloride (pH 6.67 and 6.60). All elements appear swollen and contain granules in active motion. No plasma reaction in spite of occasional (Fig. 8, H) discharge by the hyaline hemocyte of cytoplasmic substance and granules. × 800.

FIGURE 10. *Gryllotalpa.* Hemolymph clot between slide and coverglass coated with three sheets of Silicone G. E. Drifilm No. 9987, 105 minutes after withdrawal from antenna. A part of the contracting discoidal gel (see text) surrounded by a ring of fluid serum. At the borderline of the clot, the last retracting fibrils are seen. Within the clot, numerous little grey patches are coagulation islands centered by hyaline hemocytes. The scale represents 200 microns. × 70.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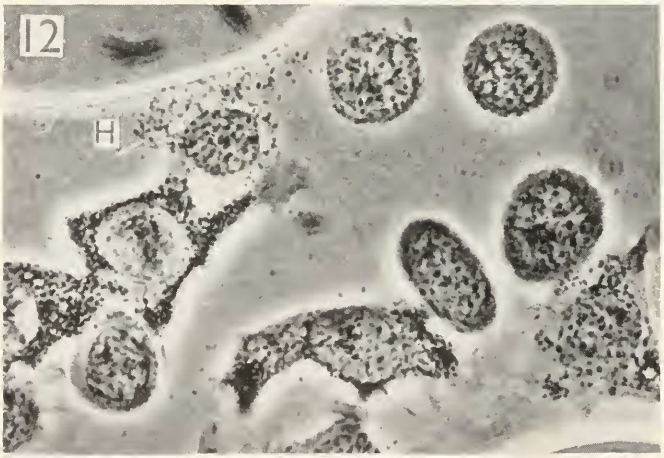
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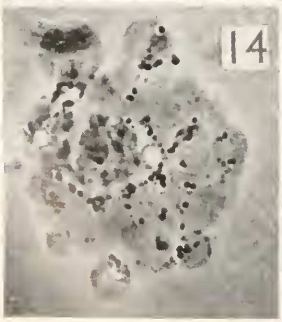
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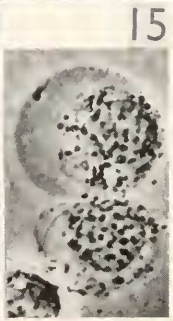
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Special reactions of the hemolymph components to the different substances tested

Differences were observed in the degree of preservation of the cytological structures of the hemocytes, when the hemolymph was mixed with solutions of the substances investigated. A satisfactory preservation of the cell integrity was obtained with solutions of potassium oxalate, sodium citrate, Sequestrene and cocaine hydrochloride. Various degrees of damage to the cytological structures were occasionally recorded with heparin, Liquoid,, Chicago Blue (2 to 15%). Solutions of sodium taurocholate (2–15%), hexamethylene-glycol (10–15%) and diethylamine (2% up) brought about dissolution of the formed elements. Milky precipitates were observed to develop frequently when the hemolymph was mixed with solutions of Liquoid, Janus green, methyl-violet and yeast nucleic acid.

In tests performed with diethylamine and protamine, the plasma reaction was characterized by the constitution of meshworks of thin elastic fibrils on which granular (precipitate) materials were absorbed.

Reaction of the hemolymph to hydrophobic surfaces

No modification nor delay in the process of coagulation was observed when the hemolymph was collected on non-adhesive surfaces of various resins and plastics (cellophane, acetophane, plexiglass, Lucite) or on glass coated with olive oil, paraffin, parlodion, formvar or with one and several sheets of silicone. Neither could coagulation be prevented when an oiled antenna was severed with oiled scissors or when the whole procedure of removing the blood was performed under oil.

When a coated coverslip was applied to the semi-spherical drop of hemolymph lying on a non-wetable slide, there was only a limited circular spreading out of the drop under the weight of the coverglass. Coagulation took place rapidly and the clot appeared as a thick elastic discoidal gel with sharp boundaries. This gel retracted progressively and within a few minutes it appeared surrounded by a ring of exuded serum (Fig. 10).

Discussion

Reliability of the procedure used

In the present study, the development or the absence of alterations in a category of hyaline hemocytes and in the plasma directly surrounding these elements was

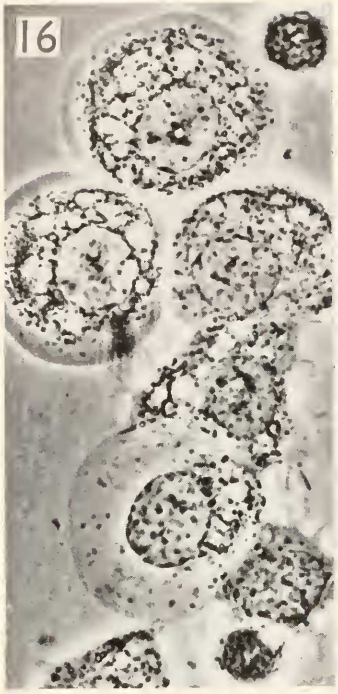
Figure 11. *Periplaneta.* Twenty-five minutes after addition of hemolymph to a 0.5% solution of potassium oxalate (pH 7.60). No plasma reaction around several hyaline hemocytes with unsharp cytoplasmic outlines or disintegrated to naked nuclei surrounded by fragments of cytoplasm. Elements belonging to other categories of hemocytes have undergone various modifications (lappet-like pseudopodia, vacuoles, cytoplasmic buddings). × 800.

Figure 12. *Periplaneta.* Twenty minutes after addition of hemolymph to a 0.1% solution of Sequestrene NA 2 (diluted from a 2% solution at pH 4.10). No plasma reaction around the hyaline hemocytes. × 800.

Figure 13. *Periplaneta.* Forty minutes after addition of hemolymph to a 0.1% solution of Belganyl-Suramin, diluted from a 2% solution at pH 7.37. A hyaline hemocyte and two granular hemocytes. No plasma reaction. × 800.

Figure 14. *Gryllotalpa.* Sixty-five minutes after addition of hemolymph to a 1% solution of Chicago Blue 6 B (pH 8.66). No plasma reaction around an altered hyaline hemocyte (peripheral cytoplasmic blisters). × 800.

Figure 15. Praying mantis (*Mantis religiosa,* Orthopt.). Forty minutes after addition of hemolymph to a 1% solution of potassium oxalate. No plasma reaction around two hyaline hemocytes. In control preparations (see Grégoire, 1951a, Figs. 9, 10 and 11) all elements of this category are surrounded rapidly by dense coagulation islands. × 800.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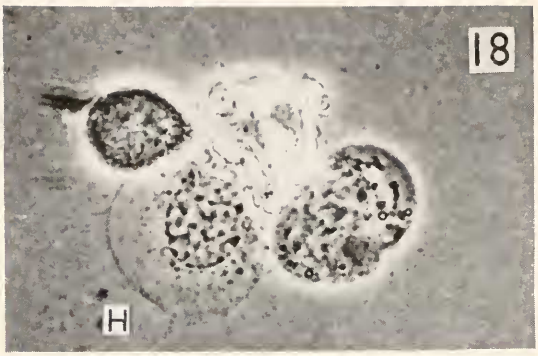
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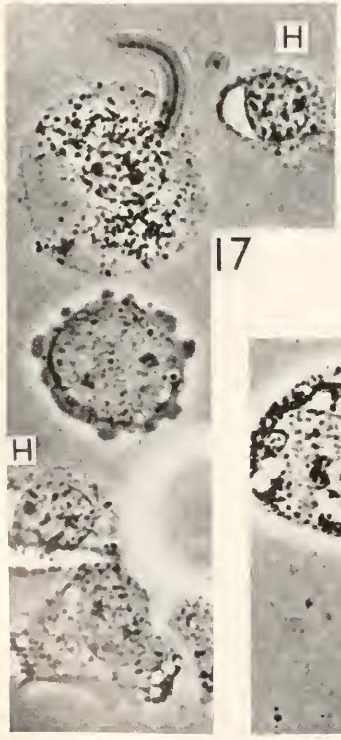
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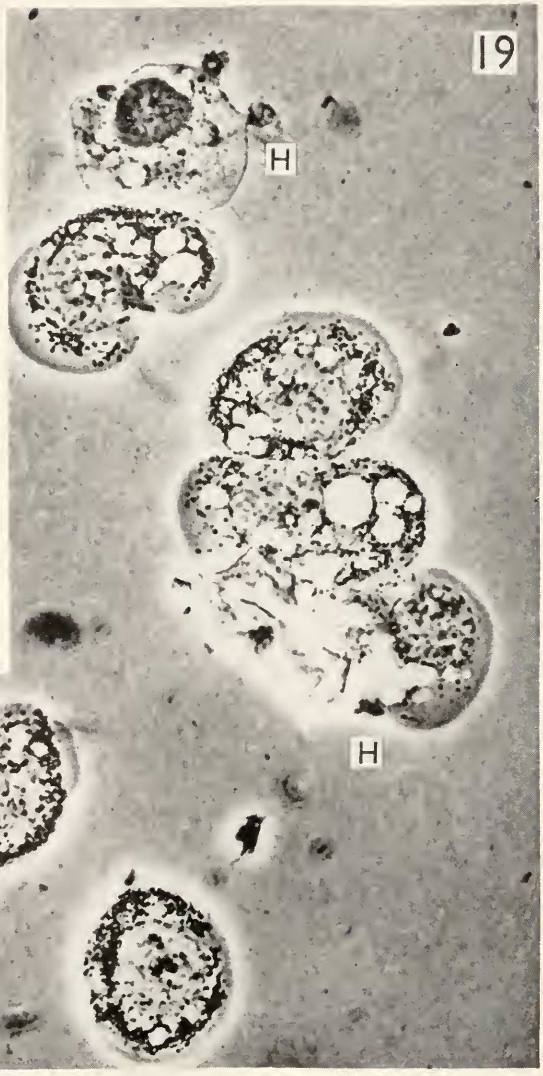
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the test selected for appreciating the anticoagulant effects of different substances on insect hemolymph.

In spite of this rather simple criterion the following pitfalls could conceivably interfere with a correct appreciation of the results:

1) The extreme speed of coagulation of shed hemolymph which characterizes different insects constitutes a serious handicap in any experimental study of the process in these animals (Beard, 1950). This property of the insect blood is related to the high degree of lability exhibited by the hyaline hemocytes (coagulocytes) to contact with foreign environment (Grégoire and Florkin, 1950; Grégoire, 1951a): in these conditions, coagulation can easily take place at the wound site before hemolymph reaches the solution of inhibitor or is thoroughly mixed with it (Beard, 1950). In the latter case, microscopic areas of normal coagulation can develop at the site of falling of the drop into the anticoagulant solution, especially when hyaline hemocytes, still bathed in their normal plasma environment, accidentally meet tiny foreign bodies, such as dust particles or pieces of chitinous debris. Dipping antennas or legs into the solution before severance, immediate shaking to insure dispersion of the formed elements and thorough contact between the labile hemocytes and the inhibitor were found to decrease the occurrence of these accidents. This procedure, however, prevents accurate evaluation of the amount of blood flowing into the solution.

2) Stirring of the mixtures by shaking before the coverglass is applied does not completely prevent local variations in the degree of plasma dilution in different areas of the film spread out between slide and coverslip. Absence of islands of coagulation around hyaline hemocytes in the areas of higher dilution, usually in the peripheral parts of the preparations, can give deceptive pictures of inhibition. Attempts to appreciate this factor were performed in mock preparations, in which solutions of colloidal dyes (*e.g.*, india ink) were used instead of hemolymph and submitted to the same mechanical agencies. The distribution under the coverglass of the areas with various densities of the diluted dye was recorded, the stirring repeated on similar tests until films of uniform tone were obtained, and the same mechanical procedure applied to the diluted hemolymph.

3) The present results have been established on standard preparations consisting mostly of approximately equal amounts of hemolymph and solutions to be tested. It is possible that decreasing the relative amount of hemolymph or increasing that of the solutions would allow one to observe anticoagulant effects for substances for which none has been recorded in the present conditions.

FIGURE 16. *Periplaneta.* One hundred and ten minutes after addition of hemolymph to a 2% solution of sodium nucleinate Merck (pH 5.50). Area without plasma coagulation (exceptional). Seven hemocytes including a swollen hyaline hemocyte. × 800.

FIGURE 17. *Periplaneta.* Sixty minutes after addition of hemolymph to a 2% solution of sodium thiosulfate in distilled water (pH 6.25). No plasma reaction around hyaline hemocytes. The large hyaline cell with moving intra-cytoplasmic granules belongs probably to the same category. × 800.

FIGURE 18. *Periplaneta.* Preparation as in Figure 17, after 70 minutes. There is no plasma reaction around the hyaline hemocyte anchored to a foreign body (piece of chitinous debris). In normal hemolymph, contact with a foreign body would rapidly induce production of a coagulation island around such a hyaline hemocyte. × 800.

FIGURE 19. *Periplaneta.* Twenty-five minutes after addition of hemolymph to a 1% solution of Liquoid (pH 7.60). No plasma reaction around two hyaline hemocytes, one of them attached to a foreign body. × 800.


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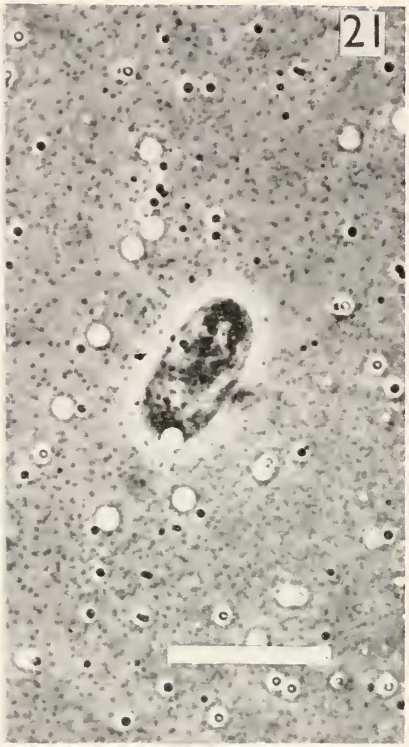
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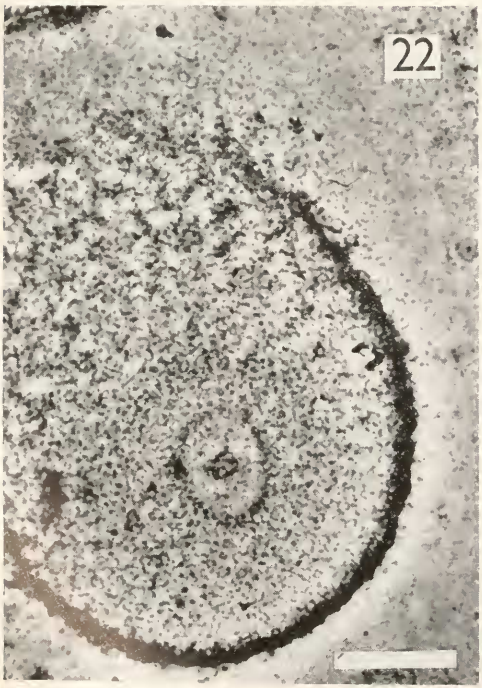
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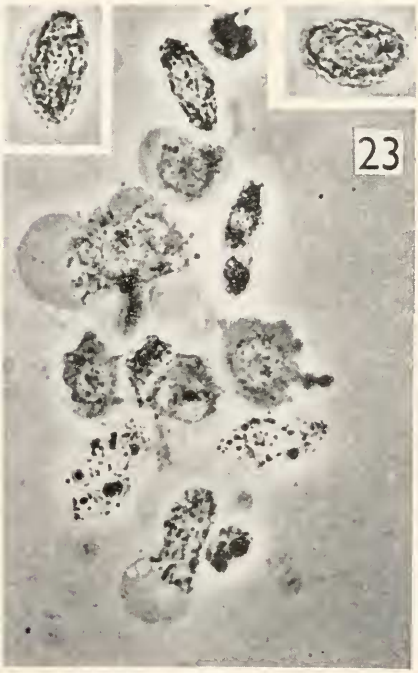
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In addition, the threshold of efficiency of various substances seems to vary with the species of insect used: for instance, as seen in different tests, for preventing coagulation in *Gryllotalpa* hemolymph, it was often necessary to use higher concentrations of anticoagulant solutions than for obtaining inhibition of the coagulation of *Periplaneta* hemolymph, with the same substance.

Comparative study of the literature

Coagulation of insect hemolymph has been reported to be prevented by various organic acids: nucleic (Paillot, 1923), citric and ascorbic acids (Beard, 1950), vapors of acetic and several fatty acids (Shull, Riley and Richardson, 1932; Fischer, 1935; Shull, 1936); also by surface-active and physical agents, such as heating of the animal body (Fredericq, 1881; Yeager, Shull and Farrar, 1932; Babers, 1938; Beard, 1950), freezing and ultrasonic waves (Beard, 1950).

On the other hand, failure of several anticoagulants of mammalian blood to inhibit the coagulation of the insect hemolymph has been reported for magnesium sulfate, (Fredericq, 1881), potassium oxalate (Muttkowski, 1924; Yeager, Shull and Farrar, 1932), heparin, hirudine, citrates, dicoumarol (*in vivo:* Beard, 1950). Hemolymph coagulation was not affected by cyanide fumes (Muttkowski, 1924; Shull, Riley and Richardson, 1932), nor by calcium cyanide (Beard, 1950).

The present results are at variance with the former data obtained with potassium oxalate, citrate and to some extent with heparin.

The discrepancies could be explained by differences in the experimental conditions (for instance, concentrations and relative amounts of the inhibitors used). As the literature on the process of normal coagulation in insects is controversial, it is more probable that the standards used for determining an anticoagulant effect cannot be compared.

The normal hemolymph coagulation of different insects has been described as being either a cellular agglutination or a plasma coagulation, both considered as two physiologically distinct processes, which can occur independently or together in the same insect or in different insects (see Beard, 1950).

In a recent paper, Beard (1950) refers to the difficulties encountered in appreciating the effects of the coagulation inhibitors on insect blood, while with the clotting of mammalian blood, fibrin formation is a convenient end-point for judging

FIGURE 20. Water scorpion (*Nepa cinerea,* Hemipt.). Undiluted hemolymph, 25 minutes after withdrawal. A large circular island of coagulation is seen surrounding an altered hyaline hemocyte. × 1080.

FIGURE 21. *Nepa.* Forty minutes after addition of hemolymph to a 1% solution of potassium oxalate (pH 8.10). A cucumber-like inert hyaline hemocyte is seen floating freely in the fluid plasma. Numerous grey and black dots are moving precipitates of calcium oxalate. × 1080.

FIGURE 22. *Lethocerus cordofanus* Mayr (Belostomatidae from the Belgian Congo). Undiluted hemolymph, 25 minutes after withdrawal. A hyaline hemocyte is seen in the center of a circular island of coagulation. × 800.

FIGURE 23. *Lethocerus.* Eighty minutes after addition of hemolymph to a 0.5% solution of Sequestrene NA 2. No plasma reaction. A group of variously altered elements belonging to different categories of hemocytes. Owing to the shrinkage, hyaline hemocytes are difficult to recognize unquestionably: two pale cells (lower part of the picture) and two elements isolated in squares are probably hyaline hemocytes. × 800.

the effect. According to Beard, dispersion of the formed elements is the desired response in searching for an inhibitory agent.

As it appears from our previous and present studies, a process of cellular agglutination does not play any part in the phenomenon of hemolymph coagulation in the species of insects used in the present experiments. The categories of blood cells other than the hyaline hemocytes are inert, scattered or agglutinated at random and passively embedded in the plasma coagulum. Accordingly, the dispersion of the formed elements could not be selected as a test for measuring an inhibiting effect on the process of hemolymph coagulation in insects.

The present results, on the other hand, are in agreement with various data obtained with crustacean blood, as regards the inhibiting effect on the true plasma coagulation (so called second coagulation) of magnesium sulfate (Halliburton, 1885; Ducceschi, 1903, 1915), potassium oxalate (Bottazzi, 1902; Ducceschi, 1903, 1915; Loeb, 1903, 1904; Nolf, 1909; Parsons and Parsons, 1923), highly concentrated solutions of peptone (Bottazzi, 1902; Loeb, 1903; Nolf, 1909; Parsons and Parsons, 1923), sodium hydrosulfite (Hensill, 1948) and cocaine hydrochloride (Ducceschi, 1903, 1915).

Mechanism of the coagulation inhibiting effect in insect hemolymph

Little is known of the chemical nature of the substances involved in the process of hemolymph coagulation in insects. The terms fibrin and gelatine used in the literature for characterizing different aspects of the insect clots rather represent morphological analogies with the corresponding compounds in crustacean or vertebrate blood.

An anticoagulant can act on insect hemolymph either on the hyaline hemocytes involved in the initiation of the process or on the coagulable compounds contained in the plasma, or on both factors.

From the present data, different anticoagulants can be efficient in preserving the extremely labile hyaline hemocytes from the alterations which they rapidly undergo in normal hemolymph when they come in contact with glass surfaces and foreign particles and which distinguish this category of blood elements from the other less labile hemocytes. In mixtures of hemolymph and efficient inhibitors, the hyaline hemocytes generally keep a spherical or oval shape and do not spread out. Inconstant anticoagulants are unable to prevent completely the alterations of all the hyaline hemocytes and the subsequent reaction of the plasma in their vicinity: the coagulation process is scattered and the areas of extension of the coagulum are decreased by various degrees.

As often suggested for a corresponding category of elements in crustacean blood (Halliburton, 1885; Hardy, 1892; Ducceschi, 1903; Tait and Gunn, 1918) the hyaline hemocytes of insects could take part in the coagulation process by yielding coagulation—inducing substances. Anticoagulants would prevent the release of these active substances. However, in incidental observations, no clotting reaction could be detected in the plasma around some hyaline hemocytes which had discharged a part of their cytoplasmic substance or were reduced to naked nuclei after their rupture. This result could be explained as well by inactivation of the coagulation-inducing substances contained in these hemocytes as by alterations of one or more plasma components involved in the coagulation process.

Of six compounds which act on vertebrate blood as removers of calcium ions, deionizing agents or preventers of platelet disintegration, five were efficient anticoagulants of insect hemolymph. The present results do not allow one to conclude whether these compounds interfere with the insect coagulation process either like other strong salts, in preventing the release of active substances by the hyaline hemocytes, or in inactivating these substances, or by removing calcium salts possibly involved in the mechanism of the clotting process.

In preliminary experiments, a calcium chloride solution was allowed to flow into a film of oxalated hemolymph. When the calcium chloride reached the spherical, inert hyaline hemocytes bathed in the oxalate solution, these elements underwent sudden modifications, consisting of shrinkage or the extension of adhesive pseudopodia. A definite reaction was not observed in the plasma directly surrounding these elements. Owing to the formation of precipitates between potassium oxalate and calcium chloride, interfering with the observations, the development of veil-like elastic structures in different areas of the preparations, suggesting a possible clotting reaction, remains questionable and requires further study.

Among the organic esters of sulfuric acid, which were found to be efficient anticoagulants of insect blood in the present study, it is worth mentioning that the trypanocidal drug suramin would be an inhibitor of enzymatic activity, according to a recent finding (Willis and Wormall, 1950).

Several other anticoagulants of vertebrate blood, listed above, were not inhibitors at the concentrations used or brought about various degrees of interference with the process of coagulation.

Owing to the lack of data on the number and on the chemical nature of the coagulation factors in insects, interpretation of the failure of these anticoagulants of vertebrate blood to inhibit the process in insects cannot be given at the present time.

Comparative physiology

The present results offer additional data with regard to the existence of converging functional characters in zoological groups as distant as arthropods and vertebrates: vertebrate platelets (Ferguson, 1934), Hardy's explosive corpuscles in crustacea (Hardy, 1892; Ducceschi, 1903, 1915; Tait and Gunn, 1918) and hyaline hemocytes in insects (Grégoire and Florkin, 1950; Grégoire, 1951a) exhibit specific morphological changes, which are related to the initiation of the plasma coagulation. Substances decreasing, delaying or preventing the alterations in the platelets (Bizzozero, 1882; Bürker, 1904; Wright, 1941; Feissly and Ludin, 1949), in the crustacean explosive corpuscles (Ducceschi, 1903, 1915; Tait and Gunn, 1918), and in the hyaline hemocytes of insects, as shown in the present studies, decrease, delay or prevent the blood coagulation. However, there are differences between vertebrate platelets and insect hyaline hemocytes; the latter elements do not exhibit, at least *in vitro*, the property of auto-agglutinability characterizing the platelets, and their fragility seems to be much greater. In the present investigations, the hyaline hemocytes were not preserved on hydrophobic surfaces, even when they were protected by oily environment from contamination with the tissues at the wound, before falling onto the silicone-coated surface. The oil procedure was successful in the hands of Tait and Gunn (1918) in preventing on plain glass

the alterations in the explosive corpuscles and the plasma coagulation in *Astacus* blood.

Consistent failure of the silicone-coated surfaces to prevent coagulation of insect hemolymph seems to indicate that different factors are probably responsible for the induction of the alterations in insect hyaline hemocytes and vertebrate platelets.

Substances tested and reactions of insect hemolymph. Summarized report of the data

For brevity's sake, the reference marks used here below record the following reactions of the plasma:

+: development of coagulation islands around hyaline hemocytes (coagulocytes), followed by various degrees of extension of the process of coagulation;

−: no coagulation, all hyaline hemocytes inert, loosely fixed to the glass or floating in the fluid plasma;

*: development of coagulation islands or veils in limited areas, obviously accidental, induced in the vicinity of foreign bodies, or at the site of falling down of the drop of hemolymph into the solution of anticoagulant (see text);

$: heterogeneous reaction. Areas without coagulation (oxalate type), areas with various degrees in the reduction of the process, as compared with the control preparation in Meisenheimer's fluid. Plasma reaction consisting of tiny coagulation islands or fringes around the hyaline hemocytes, and of thin veils without or with poor extension.

The concentrations of the solutions used are given in %. Unless otherwise indicated, the pH values are those of solutions in Meisenheimer's fluid. The preparations consisted of approximately equal volumes of solution investigated and of hemolymph. The total volume of mixture in each test amounted approximately 0.005 to 0.01 cc. The mixtures were spread out into films under 24 × 24 mm. and 24 × 30 mm. coverslips (see methods above).

1. *Controls of the factors dilution and pH of the solutions of the substances investigated.*

Meisenheimer's fluid (incidentally distilled water).

pH 0.6, 1.20, 3.30, 4.12, 4.32, 5.63, 5.83, 7.28, 7.38, 7.83, 8.00, 9.45: 160 tests, all +.

2. *Salts, removers of calcium ions and deionizing agents.*

Potassium oxalate.

0.07%: 3 tests $ and 1 test − *.
0.1%: 2 tests $ and 2 tests − *.
0.2%: 8 tests −.
0.5%, 1% (pH 7.60–8.10), 4%, 10% (pH 7.80–8.00) and 20%: 32 tests −.

Sodium citrate.

0.1%: 3 tests $ and 2 tests −.
0.3%: 3 tests $ and 3 tests − (incidentally *).
0.4%: 2 tests $ and 6 tests − (incidentally *).
1% (pH 7.58): 14 tests − (incidentally *).

Magnesium sulfate.
Saturation (pH 5.86) : 5 tests −.
Sequestrene NA 2.
0.1%, 0.5%, 1% and 2% (pH 4.10) : 23 tests − (incidentally *).
Saccharose (in Meisenheimer's fluid).
2%, 5%, 10% (pH 7.21) and 50% (pH 7.27) : 7 tests + and 5 tests $.
Saccharose (in distilled water).
1% (pH 6.57), 10% (pH 6.40) and 50% (pH 6.20) : 8 tests + and 12 tests $.
Tricalcium phosphate.
1% (pH 6.51) : 2 tests +.

3. *Organic esters of sulfuric acid.*

Heparin (*Liquemine* Roche).
50 anticoagulant units per drop used (pH 6.50) : 3 tests +, 17 tests $ and 10 tests −.
Heparin (powder dissolved in Meisenheimer's fluid).
4800 Toronto anticoagulant units per cc.: 5 tests $ and 3 tests −.
2400 Toronto anticoagulant units per cc.: 2 tests +.
Treburon Ro 2–3053/B–3.
1% (*Periplaneta*) : 6 tests − (incidentally *).
2% (*Periplaneta*) : 6 tests − (incidentally *).
1.5% (*Gryllotalpa*) : 1 test $ or − and 1 test −.
3.75%, 7.5% and 15% (*Gryllotalpa* and *Blabera*) : 16 tests − (incidentally *).
Suramin (Belganyl).
0.01% : 2 tests $.
0.1%, 0.2% (in distilled water: pH 6.26), 2 % (in Meisenheimer's fluid: pH 7.37) and 10% (in distilled water: pH 6.20) : 12 tests −.
Bayer 205 (*Germanine*).
0.02% : 2 tests + (*Gryllotalpa*) and 4 tests $ (*Periplaneta*).
0.2% and 2% (pH 7.50) : 12 tests −.
Chicago Blue 6 B.
0.1% : 4 tests + (*Gryllotalpa*) and 4 tests − (*Periplaneta*).
1% (pH 8.66) and 10% (pH 8.61) : 18 tests −.
Liquoid.
0.01%, 0.02% (pH 7.65), 0.1% (in distilled water: pH 6.43 and in Meisenheimer's fluid: pH 7.82–7.96) : 11 tests +.
0.5% : 2 tests $ and 2 tests −.
1% (pH 7.60) : 6 tests −.
2% (in distilled water: pH 6.40) : precipitates, 3 tests −.
Chlorazol fast pink BKS.
0.005% : 2 tests +.
0.01%, 0.02%, 0.05%, 0.2%, 0.5% and 2% (pH 6.00) : 7 tests $ and 21 tests − (precipitates).

4. *Organic bases and basic dyes.*

Protamine.
0.1% and 1% (in distilled water: pH 1.83) : 8 tests +.
Diethylamine.
0.1% (in distilled water: pH 9.29) : 6 tests + and 2 tests −.
0.5% (in distilled water: pH 10.61) : 6 tests $.
1% (in distilled water: pH 11.07 and in Meisenheimer's fluid: pH 11.83) : 2 tests $, 8 tests −. In 2 other tests, dissolution of the hemocytes.
1% (acidified to pH 6.00) : 2 tests +, 4 tests $ and 2 tests −.
10% (pH 12.27) : 3 tests $ and dissolution of the hemocytes.
10% (acidified to pH 5.80) : 8 tests −.
Methylene Blue.
0.02% and 0.1% (pH 7.38) : 2 tests +, 8 tests $ and 2 tests − *.
1% (pH 3.30) : 4 tests $ and 2 tests −.
2% (pH 3.30) : 6 tests −.
Janus Green B.
0.02% : 2 tests $ (*Gryllotalpa*) and 6 tests − (*Periplaneta*).
0.2% (in distilled water: pH 3.89 and in Meisenheimer's fluid: pH 5.38) and 2% (pH 2.66) : 9 tests − and precipitates.
Methyl violet.
0.1% and 0.3% : 2 tests +, 1 test $ and 5 tests −.
1% (pH 6.62) : 2 tests: precipitates.

5. *Reducing substances.*

Sodium bisulfite.
2% (pH 4.49) and 5% (pH 4.30) : 8 tests −.
Sodium thiosulfate.
2% (pH 6.25) and 10% (pH 5.92) : 8 tests − (in 2 tests *).
Sodium hydrosulfite.
3% (pH 5.50) : 2 tests −.
Cysteine hydrochloride.
0.2% and 1% : 3 tests +.
2% (pH 1.10) : 5 tests + and 6 tests $.
2% (alkalinized to pH 8.30) : 8 tests − (in 4 tests *).
10% (pH 0.60) : 5 tests +, 2 tests $ and 2 tests −.
10% (alkalinized to pH 6.92) : 8 tests−.
Glutathione.
0.2% and 1% : 3 tests + and 1 test $.
2% (pH 2.70–2.85) : 3 tests + (*Periplaneta*), 10 tests $ (*Periplaneta*) and 8 tests − (*Gryllotalpa*).
2% (alkalinized to pH 7.30) : 8 tests + (*Gryllotalpa*), 8 tests $ (*Periplaneta, Gryllotalpa*) and 8 tests − (*Periplaneta*).
10% (pH 2.61) : 2 tests + and 5 tests $.
10% (alkalinized to pH 8.79) : 4 tests −.
l-ascorbic acid cryst.
2% (pH 2.90) : 2 tests $ and 8 tests − *.
10% (pH 2.20) : 5 tests $ and 3 tests −.

6. *Salts of rare earth metals.*

Lanthanum chloride.

0.2% and 1%: 10 tests + (precipitates).
5%: 2 tests $ and 2 tests − *.
10%: 6 tests − (incidentally *).

7. *Miscellaneous substances.*

Peptone (Merck 7213).

2% (in distilled water: pH 7.34 and in Meisenheimer's fluid: pH 7.66): 10 tests $.
5%: 2 tests −.
10% (in distilled water: pH 7.58 and in Meisenheimer's fluid: pH 7.38): 4 tests $ and 9 tests −.
16% (pH 7.60): 5 tests −.

Sodium taurocholate.

0.1%, 0.5%, 2% (pH 7.00–7.17), 5% (pH 7.02), and 10%: 3 tests + and 20 tests $.
16% (pH 6.99): 10 tests − (dissolution of the hemocytes).

Cocain hydrochloride.

1% (pH 6.17 − 6.67) and 5%: 18 tests − (incidentally *).

Sodium nucleinate (Merck).

2% (pH 5.48) and 10% (pH 4.99): 6 tests + and 4 tests $.

Yeast nucleic acid (Merck).

0.025%, 0.5%, 1%, 2% (pH 3.00) and 10% (pH 3.48): 13 tests + and in 1 test, precipitates.

Apikur (bee venom).

0.015%, 0.03% and 0.07%: 6 tests +, 9 tests $ and 1 test −.
0.15% (pH 3.40): 8 tests $ and 1 test −.

Hexamethylene glycol.

2% (in distilled water: pH 5.60 and in Meisenheimer's fluid: pH 6.96), 5% (in distilled water: pH 5.50 and in Meisenheimer's fluid: pH 6.96): 20 tests + and 2 tests $.
10% (pH 6.78): 3 tests $ and 9 tests − (incidentally *).
15% (pH 7.27): dissolution of the hemocytes in all tests.

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10%: 8 tests − (incidentally *).

Summary

1. The effects of 33 substances, most of them anticoagulants of vertebrate blood, have been investigated *in vitro* on the hemolymph of different species of insects, chiefly Orthoptera, exhibiting in normal conditions a conspicuous process of coagulation.

2. The tests (900 approximately) consisted of mixtures in equal amounts of hemolymph and solutions at various concentrations in a special Ringer for insects (Meisenheimer's fluid) of the substances studied. The mixtures were observed by means of a phase contrast microscope after they were spread out in thin films between slide and coverglass.

3. The part played in the reactions by the dilution of the hemolymph and by the pH of the solutions used was appreciated in control preparations made with Meisenheimer's fluid alone at various pH values; no interference with the coagulation process was recorded within the range of pH used (0.60 to 9.45).

4. The morphological criterion selected for appreciating an anticoagulant effect was the development or the absence of alterations in the category of hemocytes especially involved in the initiation of the plasma coagulation, and the development or the absence of coagulation islands around these cells.

5. From the 33 substances tested, 18 were efficient anticoagulants in relatively small amounts; among them were four salts, removers of calcium ions or de-ionizing agents of vertebrate blood, six organic esters of sulfuric acid, including trypanocidal drugs, two basic dyes and three reducing substances. Consistent anticoagulant effects could not be obtained with heparin, and with solutions of the following substances (unless at high concentrations and, for some of them, after acidification or alkalinization): diethylamine, cysteine hydrochloride, glutathione, l-ascorbic acid, peptone, Apikur and hexamethylene glycol.

6. Different hydrophobic surfaces, including glass coated with Silicone G. E. Drifilm No. 9987, did not induce any definite modification in the coagulation process.

7. A tentative explanation of the anticoagulant effects is given in the discussion; when the substances tested prevent the morphological alterations in the category of hyaline hemocytes involved in the process of coagulation, this process does not occur. The results bring additional support to the previous interpretation that the hyaline hemocytes play an important part in the initiation of the hemolymph coagulation in different insects.

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